MOLECULES FOR MODULATING ACTIVITY OF TOLL-LIKE RECEPTORS AND METHODS OF USING THE SAME

RELATED APPLICATION

[001] This patent application claims priority to, and the benefit of, United States provisional patent application serial number 60/513,231, filed 21 October 2003, entitled, "Toll-Like Receptor Binding Peptides and Methods of Use," which application is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[002] This invention relates to molecules that interact with a Toll-like receptor (TLR), compositions comprising such molecules, and methods of using such molecules. A preferred class of such molecules are peptides that interact with one or more animal TLRs. Such molecules can be used, for example, to modulate inflammatory responses in immune-mediated diseases, ranging from autoimmunity to cancer to infectious diseases. More specifically, this invention relates to selecting heat shock proteins (HSP)-derived peptides capable of interfering with or modulating (*e.g.*, inhibiting) pro-inflammatory responses resulting from and/or related to the interaction between HSPs and derivatives thereof and TLRs on immune cells.

BACKGROUND OF THE INVENTION

1. Introduction.

[003] The following description includes information that may be useful in understanding the present invention. It is not an admission that any such information is prior art, or relevant, to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

2. Background.

Heat Shock proteins and Toll Like Receptors

[004] HSPs are highly conserved molecules that act as chaperones involved in the folding of newly synthesized proteins. In addition, they transport antigens and, after internalization, they mediate antigen-specific cytotoxic T cell, as well as Th cell-dependent, responses. During stress, synthesis of HSPs is up-regulated in prokaryotic, as well as in eukaryotic, cells. Moreover, intracellular redistribution of HSPs, and the expression of these molecules on the cell surface, has been reported.

[005] Recently, the stimulatory capacity of extracellular HSP60 and HSP70 on the innate immune system has been recognized. Human and murine macrophages respond to both bacterial and human HSP60 with the release of proinflammatory mediators such as TNF or IL-6 and of the Th1-promoting cytokines IL-12 and IL-15, suggesting that HSP60 might act as a "danger signal" for the innate immune system. The cell surface receptor for HSP60 has not yet been completely characterized. Increased local expression of HSP60, and presumably other HSPs, in target tissues has been found in various inflammatory diseases such as rheumatoid arthritis, insulitis, and atherosclerosis. It has been reported that HSP60 is an important target for immune responses during chronic inflammation or atherosclerosis T cell responses to autologous HSP60 therefore have been suggested to regulate inflammatory or auto-aggressive immune reactivities.

[006] There is evidence that CD14 and TLR 2 and 4 are involved in HSP60-mediated cell activation. However, binding to macrophages appears to be TLR4-independent. Recently, a report on HSP60-induced signaling pathways in macrophages has been published, suggesting that the stress-activated protein kinases c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), as well as I B kinase, may be activated after endocytosis of HSP60 by murine macrophage. These kinases induce transcription factors like NF-KB that are responsible for cytokine (e.g., TNF) gene transcription.

[007] Interestingly, HSP60 and 70 are also antigens for the adaptive immune system, and T cell responses to epitopes from these HSPs regulate inflammatory diseases like rheumatoid arthritis, insulin dependent diabetes mellitus, and artherosclerosis.

[008] Indeed, autologous HSP60 has been suggested to control the balance of T cell responses in inflammation and autoimmunity. Activation of T cells is induced through their interaction with antigen presenting cells (APCs), for example, dendritic cells (DCs). DCs reside in an immature form in non-lymphoid tissues where they efficiently capture foreign antigens. Upon activation by pathogens or by inflammatory stimuli like TNF or IL-1, DCs migrate to lymphoid organs where they potently activate antigen-specific T cell responses. During migration, DCs undergo functional and phenotypic changes termed as "maturation." For example, they up-regulate surface molecules like MHC class I and II molecules, CD86, CD40, and CD54, and, thus, become potent inducers of T cell activation. Maturation is further driven by CD40-CD40 ligand interaction upon contact of DCs with T cells. In parallel, DCs lose their capacity for endocytosis and de novo MHC class II synthesis during migration to the lymphoid tissues. This feature enables DCs to preserve the antigen taken up in the periphery for antigen-specific T cell activation. In addition, mature DCs are able to release large amounts of proinflammatory cytokines like TNF or IL-1 and of the Th1-promoting cytokines IL-12 and IL-18 but they also secrete counter regulatory IL-10. Therefore, DCs are not only unique in induction of naive T cell activation, but also play a decisive role in Th cell polarization.

[009] Turning now to TLR subject matter, recognition of pathogens is also mediated by a set of germ line-encoded receptors that are referred to as pattern-recognition receptors (PRRs). These receptors recognize conserved molecular patterns (pathogen-associated molecular patterns), which are shared by large groups of microorganisms. It has been discovered that TLRs function as the PRRs in mammals and play an essential role in the recognition of microbial components. The TLRs may also recognize endogenous ligands induced during the inflammatory response. Similar cytoplasmic domains may allow TLRs to use the same signaling molecules used by the interleukin 1 receptors (IL-1Rs); these include MyD88, IL-1R–associated protein kinase and tumor necrosis factor receptor–activated factor 6. Evidence is accumulating that the signaling pathways associated with each TLR are not all identical and may, therefore, result in different biological responses. Thus, there has been no disclosure of using TLRs in a specific and deliberate manner to affect disease states in the medical field.

[0010] Therefore, there is a need in the art for inventions such as that described herein, which provides a use for peptides derived from various proteins, *e.g.*, HSP60, to interact with TLRs for the medical purpose of modulating immune response in patients.

3. Definitions.

[0011] Before describing the instant invention in detail, several terms used in the context of the present invention will be defined. In addition to these terms, others are defined elsewhere in the specification, as necessary. Unless otherwise expressly defined herein, terms of art used in this specification will have their art-recognized meanings.

[0012] The term "amino acid" denotes a molecule or residue thereof containing an amino group and a carboxylic acid group. Amino acids can be naturally occurring and non-naturally occurring amino acids, as well as any modified amino acid that may be synthesized or, alternatively, obtained from a natural source.

[0013] The terms "contacting", "combining" reagents to "form a reaction mixture", and the like mean that the various reagents and reactants required for a particular reaction are brought together under conditions that allow the reaction to occur. For example, in the context of TLR interactions with molecules of the invention, "contacting" or "combining" means that TLR molecules and molecules that act as TLR agonists or antagonists are brought together under conditions that allow the agonist or antagonist molecules to specifically interact with TLR molecules.

[0014] In the context of this invention, a "liquid composition" refers to one that, in its filled and finished form as provided from a manufacturer to an end user (*e.g.*, a doctor or nurse), is a liquid or solution, as opposed to a solid. Here, "solid" refers to compositions that are not liquids or solutions. For example, solids include dried compositions prepared by lyophilization, freeze-drying, precipitation, and similar procedures.

[0015] The term "molecule" refers to an organic chemical, including, for example, peptides, peptidomimetics, and other organic molecules. A "synthetic" molecule refers to a non-naturally occurring molecule that is made by man, *i.e.*, it does not

occur in nature in the particular active form represented. For example, a synthetic molecule that comprises a peptide refers to a molecule that contains a peptide that, by itself, does not occur in nature in active form, although the peptide may occur in nature as part of a larger molecule, *e.g.*, as part of a protein.

[0016] A "patentable" composition, process, machine, or article of manufacture according to the invention means that the subject matter satisfies all statutory requirements for patentability at the time the analysis is performed. For example, with regard to novelty, non-obviousness, or the like, if later investigation reveals that one or more claims encompass one or more embodiments that would negate novelty, non-obviousness, *etc.*, the claim(s), being limited by definition to "patentable" embodiments, specifically exclude the unpatentable embodiment(s). Also, the claims appended hereto are to be interpreted both to provide the broadest reasonable scope, as well as to preserve their validity. Furthermore, if one or more of the statutory requirements for patentability are amended or if the standards change for assessing whether a particular statutory requirement for patentability is satisfied from the time this application is filed or issues as a patent to a time the validity of one or more of the appended claims is questioned, the claims are to be interpreted in a way that (1) preserves their validity and (2) provides the broadest reasonable interpretation under the circumstances.

[0017] A "peptide" means a polymer of two or more amino acid residues linked via peptide bonds.

[0018] A "peptidomimetic" refers to a synthetic molecule that mimics one or more biological activities of a peptide according to the invention. In the context of the invention, a peptidomimetic is a molecule that specifically interacts with a TLR, *i.e.*, it is a TLR agonist or antagonist. Preferably, a peptidomimetic will possess at least about 1%, 5%, 10%, 25%, 50%, 75%, 80%, 85%, 90%, 95%, 99%, 100%, or more of the TLR binding and/or modulating activity of the peptide upon which the particular peptidomimetic is based or derived. Peptidomimetics may provide various advantages over peptides, for example, chemical stability in the gastrointestinal tract, which can facilitate oral administration of a composition comprising the peptidomimetic.

[0019] The term "pharmaceutically acceptable salt" refers to salts which retain the biological effectiveness and properties of the compounds of this invention and which are not biologically or otherwise undesirable. In many cases, the compounds of this invention are capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto. Pharmaceutically acceptable acid addition salts may be prepared from inorganic and organic acids, while pharmaceutically acceptable base addition salts can be prepared from inorganic and organic bases. For a review of pharmaceutically acceptable salts see Berge, *et al.* ((1977) *J. Pharm. Sci.*, vol. 66, 1).

[0020] A "plurality" means more than one.

[0021] The terms "separated", "purified", "isolated", and the like mean that one or more components of a sample contained in a sample-holding vessel are or have been physically removed from, or diluted in the presence of, one or more other sample components present in the vessel. Sample components that may be removed or diluted during a separating or purifying step include, chemical reaction products, unreacted chemicals, proteins, carbohydrates, lipids, and unbound molecules.

[0022] "Specifically associate", "specific association," and the like refer to a specific, non-random interaction between two molecules, which interaction depends on the presence of structural, hydrophobic/hydrophilic, and/or electrostatic features that allow appropriate chemical or molecular interactions between the molecules.

[0023] Herein, "stable" refers to an interaction between two molecules (*e.g.*, a peptide and a TLR molecule) that is sufficiently stable such that the molecules can be maintained for the desired purpose or manipulation. For example, a "stable" interaction between a peptide and a TLR molecule refers to one wherein the peptide becomes and remains associated with a TLR molecule for a period sufficient to achieve the desired effect.

[0024] A "subject" or "patient" refers to an animal in need of treatment that can be effected by molecules of the invention. Animals that can be treated in accordance with the invention include vertebrates, with mammals such as bovine, canine, equine, feline, ovine, porcine, and primate (including humans and non-humans primates) animals being particularly preferred examples.

[0025] A "therapeutically effective amount" refers to an amount of an active ingredient, *e.g.*, a peptide or other TLR-binding molecule according to the invention, sufficient to effect treatment when administered to a subject in need of such treatment. In the context of immunology, a "therapeutically effective amount" is one that produces an objectively measured change in one or more immunological parameters, including an increase or decrease in cytokine expression, expansion of one or more classes of B or T cells, production of antibodies, immunity against a pathogen, *e.g.*, adenovirus. Accordingly, what constitutes a therapeutically effective amount of a TLR-binding molecule according to the invention may be readily determined by one of ordinary skill in the art. Of course, the therapeutically effective amount will vary depending upon the particular subject and condition being treated, the weight and age of the subject, the severity of the disease condition, the particular compound chosen, the dosing regimen to be followed, timing of administration, the manner of administration and the like, all of which can readily be determined by one of ordinary skill in the art.

[0026] The term "treatment" or "treating" means any treatment of a disease or disorder, including preventing or protecting against the disease or disorder (that is, causing the clinical symptoms not to develop); inhibiting the disease or disorder (*i.e.*, arresting or suppressing the development of clinical symptoms; and/or relieving the disease or disorder (*i.e.*, causing the regression of clinical symptoms). As will be appreciated, it is not always possible to distinguish between "preventing" and "suppressing" a disease or disorder since the ultimate inductive event or events may be unknown or latent. Accordingly, the term "prophylaxis" will be understood to constitute a type of "treatment" that encompasses both "preventing" and "suppressing". The term "protection" thus includes "prophylaxis".

SUMMARY OF THE INVENTION

[0027] Accordingly, the objects of this invention include the provision of TLR-binding molecules, compositions containing them, and methods for their use. Representative examples of such molecules include peptides (produced by recombinant or synthetic techniques) and peptidomimetics. The molecules of the invention have the following characteristics: they specifically interact with the

extracellular portion of a TLR; and they exhibit agonist or antagonist activity upon binding to a TLR.

[0028] In a first aspect, isolated TLR-binding peptides, peptidomimetics, and other synthetic organic molecules are provided. In certain preferred embodiments, TLR-binding molecules are peptides that comprise core TLR-binding motifs. In further related embodiments, these motifs comprise amino acid sequences of human and bacterial HSP (*e.g.*, hsp60) proteins. The peptides of the invention provide isolated polypeptides having at least about 70% sequence identity, preferably at least about 80% sequence identity, more preferably at least about 90% sequence identity, and most preferably at least about 95% sequence identity with a TLR-binding peptide that comprises an amino acid sequence according to any of the sequences of SEQ ID NOS:1-73.

[010] In a second aspect, the invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a peptide having at least about 70% sequence identity, preferably at least about 80% sequence identity, more preferably at least about 90% sequence identity, and most preferably at least about 95% sequence identity with a TLR-binding peptide of the invention. Of course, the peptides of the invention can be made by other techniques, including peptide synthetic chemistries (e.g., solid state peptide synthesis).

[011] In a third aspect, the invention provides for TLR-binding peptide mimetic molecules useful in modulating the natural activity of the TLR receptor in immune regulation. Relatedly, the peptides and mimetics of the invention can be incorporated into various compositions, including pharmaceutical compositions wherein a peptide or peptide mimetic is combined with a pharmaceutically acceptable carrier. Such compositions can be liquid or dry, and can optionally include excipients and other molecules.

[012] In a fourth aspect, the TLR-binding peptides and mimetic molecules of the invention are useful in methods of modulating immune responses in immune-mediated diseases. For example, the peptides and mimetic molecules of the invention can be used in immune modulation and thus can provide for enhancing innate immune responses, such as, for example, inducing or enhancing inflammatory responses. With respect to such embodiments, the enhancement can be used, for example, to

provide for clearance of infectious agents, cancer cells, or to induce or enhance response to a vaccine. Alternatively, the peptides can be used as competitive inhibitors of a natural ligand for a TLR. In this regard, the peptides and mimetic molecules of the invention can be used to bring about down regulation of an immune pathway. For example, down regulation can comprise down regulation in autoimmunity such as in Rheumatoid Arthritis (RA), Juvenile Idiopathic Arthritis (JIA), Psoratic Arthiritis (PA), Osteoarthritis (OA), Inflammatory Bowel Disease (IBD), Multiple Sclerosis (MS), and Dermato Myositis (DM).

[013] The peptides and peptide mimetics of the invention are also contemplated to be used to treat cancerous conditions such as melanoma, leukemia, lymphoma, solid tumours (lung, liver, kidney, brain, bladder), retinoblastoma, sarcomas and other connective tissue cancers, and the like. The molecules of the invention are also contemplated for use in treating immune-mediated pathogenic infections including such immune mediated microbial infections as tuberculosis, leprosis, bacterial infections of Gram positive and Gram negative microroganisms, HIV/AIDS, Epstein BarrVirus and Cytomegalovirus infections, and protozoan infections, such as Leishmania, and the like. Use of the peptides and mimetics of the invention are also contemplated in Insulin-Dependent Diabetes Mellitus (IDDM), Systemic Lupus Erythematosus (SLE), Sjogren's Syndrome, Scleroderma, Polymyositis, Chronic Active Hepatitis, Mixed Connective Tissue Disease, Primary Biliary Cirrhosis, Pernicious Anemia, Autoimmune Thyroiditis, Idiopathic Addison's Disease, Vitiligo, Gluten-Sensitive Enteropathy, Graves' Disease, Myasthenia Gravis, Autoimmune Neutropenia, Idiopathic Thrombocytopenia Purpura, cirrhosis, pemphigus vulgaris, autoimmune infertility, Goodpasture's Disease, Bullous Pemphigoid, Discoid Lupus, Ulcerative Colitis, and Dense Deposit Disease). The diseases set forth above, as referred to herein, include those exhibited by animal models for such diseases, such as, for example Non-Obese Diabetic (NOD) mice for IDDM and Experimental Autoimmune Encephalomyelitis (EAE) mice for multiple sclerosis.

[014] In a fifth aspect, the peptides and mimetics of the invention can be used to modulate specific immune responses such as a TH-1 response (i.e., a stimulating response wherein proinflammatory cytokines are induced, such as TNFα and INFγ, IL-6, IL-2, IL-12), or the T cells that have a regulatory function are induced to bring

about either a TH2 or TH3 response (i.e., a regulatory response wherein anti-inflammatory cytokines are induced, such as TGFβ, IL-4, and IL-10).

[015] In a sixth aspect, the peptides and mimetics of the invention in some form affect, or alternatively are associated with, activities, or lack thereof, of cytokines and other immune mediators, including the following:

Pro-inflammatory responses

[016] IL-1a/b (interleukin 1); TNF-α (tumor necrosis factor alpha); LT (lympotoxin); IL-6 (interleukin6); GM-CSF (granulocyte macrophage colony stimulating factor); M-CSF (macrophage colony stimulating factor); LIF (leucocyte inhibitory factor); Oncostatin M (oncostatin M); IL-2 (interleukin 2); IL-3 (interleukin 3); IL-7 (interleukin 7); IL-9 (interleukin 9); IL-12 (interleukin12); IL-15 (interleukin 15); IFNα/β (interferon alpha/beta); IFN-γ (interferon gamma); IL-17 (interleukin 17); IL-18 (interleukin 18).

Immunoregulatory responses

[017] IL-4 (interleukin 4); IL-10 (interleukin 10); IL-11 (interleukin 11); IL-13 (interleukin 13); TGF-β (transforming growth factor beta).

Chemokines responses

[018] IL-8 (interleukin 8); Groa (melanoma growth stimulating activity); MIP-1 (macrophage

[019] inflammatory protein); MCP-1 (monocyte chemoattractant protein); ENA-78 (epithelial

[020] neutrophil activating peptide 78); RANTES (regulated upon activatioin T cell expressed & secreted).

Mitogens

[021] FGF (Fibroblast growth factor); PDGF (Platelet-derived growth factor); VEGF (vascular

[022] endothelial growth factor).

[023] In a related aspect, such cytokines and immune mediators can be blocked by TLR-binding peptides of the invention.

[024] In still another aspect, the invention also provides a novel methodology for determining likely TLR-binding peptide motifs by performing *in silico* computer modeling comprising methods based on, among other factors, the sequence and structure of the peptides of the invention.

[025] In a further embodiment, the peptides and mimetics of the invention can be used in the production of a medicament for use in treatments of disease states such as those benefited by either up or down regulation of inflammatory immune responses, such as those disease states disclosed herein.

[026] In yet further embodiments, the peptides and mimetics of the invention are used in diagnostic and in therapeutic applications for patients with autoimmune diseases including cancers and infectious diseases.

[027] Other features and advantages of the invention will be apparent from the following drawings, detailed description, and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[027] This patent or application file contains at least one figure executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided upon request and payment of the necessary fee.

[028] Figure 1 shows an Antigenic Index graph that shows regions of the *E. coli* HSP60 protein predicted to be exposed on the protein surface plotted above the line and unexposed regions below the line.

[029] Figure 2 shows a Kyte/Doolittle hydrophilicity graph showing average hydrophilicity values above the axis and hydrophobic regions below the line.

[030] Figure 3 shows Surface Probability wherein plots above the line approaching a value of 1 represents a hexapeptide centered at such point is predicted to have exposure at the surface of the protein.

[031] Figure 4 shows a Chou-Fasman chart indicating regions of helix vs. sheets and turns.

[032] Figure 5 shows an Antigenic Index which plot is smoothed by the refinement of the invention.

[033] Figure 6 shows a Hydrophilicity plot which plot is smoothed by the refinement of the invention.

[034] Figure 7 shows a Surface Probability plot which plot is smoothed by the refinement of the invention.

[035] Figure 8 shows a Chou-Fasman plot which plot is smoothed by the refinement of the invention.

[036] Figure 9 is a three-dimensional stick model of HSP60 from *E. coli*. A predicted surface region is noted in yellow corresponding the region 426-444 of the *E. coli* HSP60 protein, one of the selected peptides.

[037] Figures 10A-D are a series of graphs showing the effect of different peptides on expression of TLR2, TLR4 receptor, and secretion of TNF-alpha and IL-6 proinflammatory cytokines, on monocyte-derived dendritic cells.

[038] Figure 11 is a graph showing the effect of different peptides on TNF-α secretion from a mouse macrophage cell line, RAW264.7. Cell preparation and stimulation for TNF-α production were seeded into flat-bottomed 96-well plates (0.1 x 10^6 cells/200ml well). After 18 hrs of incubation, cells were stimulated with the different peptides and, as a control, LPS was used. Supernatants were harvested respectively at 6, 24, 48, and 72 hours. ELISA assay was used and the amount of TNF-alpha in culture supernatants was quantified using a Mouse TNF-α Enzyme-Linked ImmunoSorbent Assay. TNF-α values were extrapolated using a standard curve. Results are expressed in picograms TNF-α/ml. Values shown were calculated by subtracting for each peptide the value of unstimulated cells. LPS data not shown.

[039] Figure 12 is a diagram showing the general interconnection of cytokine stimulation/expression between innate and adaptive immunity pathway.

[040] Figure 13 is a flow chart outlining the experimental pathway for Examples 2 and 3, below.

[041] Figure 14 is a graph showing TNF-α production in dendritic cells using real time PCR.

[042] Figures 15A-B are graphs showing percentage of CD8+ cells and TNF-α production in dendritic cells (CD83+, HLA-DR+) measured with staining.

[043] As those in the art will appreciate, the following detailed description describes certain preferred embodiments of the invention in detail, and is thus only representative and does not depict the actual scope of the invention. Before describing the present invention in detail, it is understood that the invention is not limited to the particular molecules (*e.g.*, peptides and peptidomimetics), TLRs, systems, and methodologies described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the invention defined by the appended claims.

DETAILED DESCRIPTION OF THE INVENTION

[044] The objects of this invention include the provision of TLR-binding molecules, compositions containing them, and methods for their use. Representative examples of such molecules include peptides (produced by recombinant or synthetic techniques) and peptidomimetics. The molecules of the invention have the following characteristics: they specifically interact with the extracellular portion of a TLR; and they exhibit agonist or antagonist activity upon binding to a TLR.

[045] In accordance with the present invention there may be employed conventional chemistry, molecular biology, microbiology, and recombinant DNA techniques within the skill of the art in order to produce the TLR-binding molecules of the invention, or nucleic acid molecules encoding certain of them. Such techniques are explained fully in the literature. *See, e.g.*, SAMBROOK et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994)]; and "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M. J. Gait ed. 1984).

[046] In a first aspect, peptides comprising core TLR-binding motifs are provided. Such motifs may be found, for example, in various heat shock proteins, including hsp-60, hsp-70, and dnaJ proteins derived from organisms including *E. coli* (e.g., strain K12), Mycobacterium tuberculosis, and humans.

Table 1

Peptide source	Amino Acid Position	Amino Acid Seq.	Peptide reference number	Seq. Id. No.
Human hsp 60	156-162	KKQSKPVTT	P22	22
	154-168	ELKKQSKPVTTPEEI	P27	27
	230-241	NTSKGQKCEFQD	P28	28
	300-320	VKAPGFGDNRKNQLKDMAIAT	P3	2
	302-314	APGFGDNRKNQLK	P29	29
	360-375	GKGDKAQIEKRIQEII	P23	23
	360-376	GKGDKAQIEKRIQEIIE	P30	30
	380-400	VTTSEYEKEKLNERLAKLSDG	P4	4
	381-398	TTSEYEKEKLNERLAKLS	P31	31
	382-396	TSEYEKEKLNERLAK	P24	24
	547-558	TEIPKEEKDPGM	P32	32
E. coli hsp 60	195-211	FDRGYLSPYFINKPETG	P33	33
	345-375	GVAQIRQQIEEATSDYDREKLQERVA KLAGG	P2	6
	346-372	VAQIRQQIEEATSDYDREKLQERVAK L	P34	34
	348-370	QIRQQIEEATSDYDREKLQERVA	P35	35
	387-396	VEMKEKKARV	P36	36
	388-396	EMKEKKAR	P25	25
	426-444	LADLRGQNEDQNVGIKVAL	P1	8
	429-439	LRGQNEDQNVG	P37	37
E. coli hsp 70	95-110	DIQQRYPHLPYQFQAS	P38	38
	96-117	IQQRYPHLPYQFQASENGLPMI	P7	1
	159-172	YFDDAQRQGTKDAA	P39	39
	249-268	IREQAGIPDRSDNRVQRELL	P8	3
	253-266	AGIPDRSDNRVQRE	P40	40
	583-596	QAIKNVDKQTQDFA	P41	41
Mycobacteria hsp 60	59-68	IELEDPYEKI	P42	42
	133-143	GAKEVETKEQI	P43	43
	135-143	KEVETKEQ	P26	26
	204-213	TDPERQEAVL	P44	44
	342-370	GRVAQIRQEIENSDSDYDREKLQERLA KL	P5	10
	343-370	RVAQIRQEIENSDSDYDREKLQERLAK L	P45	45
	346-367	QIRQEIENSDSDYDREKLQERL	P12	11
	346-368	QIRQEIENSDSDYDREKLQERLA	P46	46
	383-402	TEVELKERKHRIEDAVRNAG	P6	12
	385-399	VELKERKHRIEDAVR	P47	47
	386-394	ELKERKHRI	P13	13
	518-529	VADKPEKEKASV	P48	48
Human hsp 70	28-41	IIANDQGNRTTPSY	P49	49
	239-261	NHFVEEFKRKHKKDISQNKRAVR	P50	50
	240-265	HFVEEFKRKHKKDISQNKRAVRRLRT	P9	5
	244-257	EFKRKHKKDISQNK	P14	14
	244-261	EFKRKHKKDISQNKRAVR	P51	51
	262-279	RLRTACERAKRTLSSSTQ	P52	52
	414-427	IKRNSTIPTKQTQI	P53	53

	490-500	ATDKSTGKANK	P54	54
	501-519	ITITNDKGRLSKEEIERMV	P10	7
	502-545	TITNDKGRLSKEEIERMVQEAEKYKA EDEVQRERVSAKNALESY	P55	55
	509-545	RLSKEEIERMVQEAEKYKAEDEVQRE RVSAKNALESY	P56	56
	509-542	RLSKEEIERMVQEAEKYKAEDEVQRE RVSAKNAL	P57	57
	510-532	LSKEEIERMVQEAEKYKAEDEVQ	P15	15
	561-575	KISEADKKKVLDKCQ	P58	58
	564-574	EADKKKVLDKC	P16	16
	580-602	WLDANTLAEKDEFEHKRKELEQV	P11	9
	586-601	LAEKDEFEHKRKELEQ	P59	59
	587-599	AEKDEFEHKRKEL	P17	17
Human dnaj	14-26	GASDEEIKRAYRR	P60	60
	18-25	EEIKRAYR	P18	18
	25-39	RRQALRYHPDKNKEP	P61	61
	38-50	EPGAEEKFKEIAE	P62	62
	54-70	VLSDPRKREIFDRYGEE	P63	63
	144-164	VNFGRSRSAQEPARKKQDPPV	P64	64
	176-203	YSGCTKKMKISHKRLNPDGKSIRNED KI	P65	65
	299-314	GEGLPLPKTPEKRGDL	P66	66
E. coli dnaj	11-35	GVSKTAEEREIRKAYKRLAMKYHPD	P67	67
	15-26	TAEEREIRKAYK	P19	19
	15-32	TAEEREIRKAYKRLAMKY	P68	68
	34-66	PDRNQGDKEAEAKFKEIKEAYEVLTD SQKRAAY	P69	69
	39-66	GDKEAEAKFKEIKEAYEVLTDSQKRA AY	P70	70
	41-54	KEAEAKFKEIKEAY	P20	20
	193-213	IKDPCNKCHGHGRVERSKTLS	P71	71
	333-346	LNERQKQLLQELQE	P72	72
	334-345	NERQKQLLQELQ	P21	21
	345-365	QESFGGPTGEHNSPRSKSFFD	P73	73

[047] *E. coli* HSP 60 peptitde sequence 345-375 (P2) and Mycobacterium tuberculosis HSP 60 peptide sequence 383-402 (P6) are synthetic in that with respect to P2, the peptide sequence differs from the "natural/wild-type" of *E. coli* HSP60 such that arginine 345 (R.345) has been substituted with glycine (G.345), and with respect to P6, lysine 402 (K.402) has been substituted with glycine (G.402).

[048] Sequencing algorithms can be used to measure homology or identity between known and unknown sequences. Such methods and algorithms are useful in identifying corresponding sequences present in other organisms as well as in the design of peptides of the invention. Homology or identity is often measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the

Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms "homology" and "identity" in the context of two or more nucleic acids, polypeptide, or peptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection.

[049] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for The test sequences relative to the reference sequence, based on the program parameters.

[050] A "comparison window," as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 8 to 10, 10 to 20, 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequence for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol* 48:443 (1970), by the search for similarity method of person & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection.

51] One example of a useful algorithm is BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) or 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length of 3, and expectations (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[052] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin & Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873 (1993)). One measure of similarity provided by BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a references sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference

nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[053] Other algorithms for determining homology or identity include, for example, in addition to a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information), ALIGN, AMAS (Analysis of Multiply Aligned Sequences), AMPS (Protein Multiple Sequence Alignment), ASSET (Aligned Segment Statistical Evaluation Tool), BANDS, BESTSCOR, BIOSCAN (Biological Sequence Comparative Analysis Node), BLIMPS (BLocks IMProved Searcher), FASTA, Intervals & Points, BMB, CLUSTAL V, CLUSTAL W, CONSENSUS, LCONSENSUS, WCONSENSUS, Smith-Waterman algorithm, DARWIN, Las Vegas algorithm, FNAT (Forced Nucleotide Alignment Tool), Framealign, Framesearch, DYNAMIC, FILTER, FSAP (Fristensky Sequence Analysis Package), GAP (Global Alignment Program), GENAL, GIBBS, GenQuest, ISSC (Sensitive Sequence Comparison), LALIGN (Local Sequence Alignment), LCP (Local Content Program), MACAW (Multiple Alignment Construction & Analysis Workbench), MAP (Multiple Alignment Program), MBLKP, MBLKN, PIMA (Pattern-Induced Multi-sequence Alignment), SAGA (Sequence Alignment by Genetic Algorithm) and WHAT-IF. Such alignment programs can also be used to screen genome databases to identify polynucleotide sequences having substantially identical sequences. A number of genome databases are available, for example, a substantial portion of the human genome is available as part of the Human Genome Sequencing Project (J. Roach, http://weber.u.Washington.edu/~roach/human_ genome_ progress2.html) (Gibbs, 1995). At least twenty-one other genomes have already been sequenced, including, for example, *M. genitalium* (Fraser *et al.*, 1995), *M. jannaschii* (Bult *et al.*, 1996), *H. influenzae* (Fleischmann *et al.*, 1995), *E. coli* (Blattner *et al.*, 1997), and yeast (*S. cerevisiae*) (Mewes *et al.*, 1997), and *D. melanogaster* (Adams *et al.*, 2000). Significant progress has also been made in sequencing the genomes of model organism, such as mouse, *C. elegans*, *Arabadopsis sp.* and *D. melanogaster*. Several databases containing genomic information annotated with some functional information are maintained by different organization, and are accessible via the internet, for example, http://wwwtigr.org/tdb; http://www.genetics.wisc.edu; http://genome-www.stanford.edu/~ball; http://hiv-

web.lanl.gov; http://www.ncbi.nlm.nih.gov; http://www.ebi.ac.uk; http://Pasteur.fr/other/biology; and www.genome.wi.mit.edu.

[054] Further, selection of HSP-derived peptides may be carried out by, such as, the following protocol wherein HSPs from various species are obtained using the Entrez retrieval system (http://www.ncbi.nlm.nih.gov/Entrez/) at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). The Entrez retrieval system allows for searching several linked publicly available databases and it provides access to the date-bases listed below. Any of these data bases may be employed in the methods of the invention, such as any of the following:

Databases

[055] -PubMed: biomedical literature

[056] -Nucleotide: sequence database (GenBank)

[057] -Protein: sequence database <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein>

[058] -Structure: three-dimensional macromolecular structures

[059] -Genome: complete genome assemblies

[060] -Books: BookShelf online books

[061] -Domains: conserved domains (CDD)

[062] -3D Domains: domains from Entrez Structure

[063] -GEO: Gene Expression Omnibus

[064] -GEO Datasets: curated GEO data sets

[065] -Journals: journals in Entrez

[066] -MeSH: medical subject headings

[067] -NCBI Web Site: NCBI Web site search

[068] -OMIM: Online Mendelian Inheritance in Man

[069] -PMC: full-text digital archive of life sciences journal literature

[070] -PopSet: population study datasets

[071] -SNP: single nucleotide polymorphisms

)72] -Taxonomy: organisms in GenBank

073] -UniGene: gene-oriented clusters of transcript sequences

[074] -UniSTS: markers and mapping data

[075] Protein sequences are obtained such as the list of the retrieved HSP amino acid sequences from various species used for selecting the peptides, such as for example:

Escherichia coli

[076] -hsp60: DEFINITION: GroEL, chaperone Hsp60, peptide-dependent ATPase, heat

[077] shockprotein [Escherichia coli K12]. VERSION: NP_418567.1 GI:16131968

[078] -hsp70: DEFINITION: heat shock protein, chaperone, member of Hsp70 protein family

[079] [Escherichia coli K12]. VERSION: NP_417021.1 GI:16130451

[080] -dnaj: DEFINITION: Chaperone protein dnaJ (Heat shock protein J) (HSP40). VERSION: P08622 GI:118719

Homo sapiens

[081] -hsp60: DEFINITION: heat shock 60kDa protein 1 (chaperonin); heat shock 60kD protein 1(chaperonin) [Homo sapiens]. VERSION: NP_002147.1 GI:4504521

[082] -hsp70: DEFINITION: Heat shock 70 kDa protein 1 (HSP70.1) (HSP70-1/HSP70-2).

[083] VERSION: P08107 GI:462325

[084] -dnaj: DEFINITION: DnaJ homolog subfamily B member 1 (Heat shock 40 kDa protein 1) (Heat shock protein 40) (HSP40) (DnaJ protein homolog 1) (HDJ-1). VERSION: P25685 GI:1706473

Mycobacterium tuberculosis

[085] -hsp60: DEFINITION: heat shock 60kDa protein 1 (chaperonin); heat shock 60kD protein 1(chaperonin) [Homo sapiens]. VERSION: NP_002147.1 GI:4504521

[086] -hsp70: DEFINITION: Heat shock 70 kDa protein 1 (HSP70.1) (HSP70-1/HSP70-2).

[087] VERSION: P08107 GI:462325

[088] Following retrieval of the sequences of interest, the retrieved sequences were imported or transferred to Mac Vector 7.1.1 Trial Version to analyze the primary structure of these proteins using algorithms contained in this software package, able to originate secondary structure predictions based on the analysis of the whole protein primary sequence. Several tools were then used including the following:

[089] -**Antigenic Index**: This index uses information that goes beyond single type analysis and the algorithm combines information from hydrophylicity, surface probability and backbone flexibility predictions along with the secondary structure predictions of other algorithms such Chou-Fasman and Gobson-Garnier. profiles originated using this algorithm identify regions of a given amino acid sequence that is predicted to be exposed to the surface and therefore that may be antigen sites. This analysis combinations is able to produce a composite prediction of the surface contour of a given protein. Regions of the protein predicted to be exposed at the protein's surface are plotted above the graph axis and vice versa non exposed regions are plotted below the graph axis (See Figs).

[090] -**Kite-Doolittle Hydrophilicity Profile**: This algorithm profiles graphs of the local hydrophilicity of a protein along its aminoacid primary sequence. The rationale is based on the assignment of a hydropathy values to each of the 20 amino acids based on some experimental of empirical measure. Then a window of size N run along the length of the protein and each hydropathy value is summed and then divided by N to obtain an average hydrophilicity for the window. Average hydrophilicity values are then plotted to assign values above the axis denote hydrophilic regions and potentially exposed to the surface of the protein. Conversely, values below the axis indicate hydrophobic regions that tend to be buried inside the molecule or inside hydrophobic environments such as membranes. The Kite-Doolittle scale is the most commonly used hydrophathy scale and its values are assigned using a combination of water-vapor transfer free energy for amino acid side chains and the preference of amino acid side chains for interior or exterior environments, with small adjustments made to the final values based on experimental experience.

[091] -**Surface Probability**: This algorithm predicts which regions of a protein are most likely to lie on the protein surface, based on the knowledge of which amino acids are more likely to be found on the protein surface of proteins of known structure. The algorithm employs a determination of solvent-accessible surface area of each residue. Residues were then classified as "buried" if their accessible surface are was smaller than 20 angstroms and "exposed" if the accessible surface area was larger than 60 angstroms. For each of the 20 amino acids information was compiled on what percent of the time the amino acids were found to be exposed or buried in a sample of proteins of known atomic structure. In order to calculate the fractional surface probability for each amino acid, MacVector sums the six fractional probabilities of the amino acids in the window and divides by six fractional probabilities of the amino acids in the selected window and divides by six to yield a running average of the fractional surface probability along the length of the protein. Any value close to 1.0 at any point in the sequence of the protein would mean that the hexapeptide centered about that point is predicted to be exposed at the surface. Conversely, values close to 0.0 will mean that the hexapeptide is buried in the interior of the protein.(32,33).

[092] -**Chou-Fasman**: This is a widely used method of secondary structure prediction based on known X-ray structures. The authors compiled statistics on the tendency of an amino acid to appear in a given secondary structure and used these statistics to assign the 20 amino acids into four classes: helix formers, helix breakers, sheet formers and sheet breakers. The algorithm then predicts the structure of proteins by locating clusters of helix- or sheet-forming residues in the amino acid sequence and applying a set or rules to determine if these clusters are significant enough to nucleate a helix or a beta sheet structure. Using the MacVector version, the prediction or conformation is treated independently of the others and each structure prediction is graphed separately.

[093] Once the above analysis is performed, the values assigned by the three above mentioned algorithms and originated score values depending of the algorithm were visualized in the form of a Data Summary Table (DST) or in the form of a Data Plot (DP) as provided in the following examples below. Additionally, in analyzing DST and DP, regions of the proteins were selected that contain structures denominated α-helix and β-turns (or loops) that are characterized by appearing on the surface of proteins and usually reversing the direction of a chain. β-turns refer to a

section of a polypeptide connecting regions of defined secondary structure. In many proteins loops contain functional residues in that they tend to be more flexible in conformational changes than helices and sheets and therefore tend to be relevant part of binding sites. Also, in case a core putative TLR-binding region was detected, normally a β-turn predicted to be hydrophilic and surface-exposed, flanking regions were selected on both sides predicted to be hydrophobic or not-surface-exposed but still predicted to be α-helix. In case the resulting prediction was not satisfactory, substitutions of hydrophilic amino acid were made with other amino acids having the appropriate characteristic.

[094] In preferred embodiments, the amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property or TLR-binding is retained. "NH_2" refers to a free amino group that may be present at the amino terminus of a peptide, although for purposes of this invention, the "amino terminus" of a peptide refers to the left-most terminal amino acid residue of the peptide when represented in writing as a linear sequence of amino acid residues regardless of the reactive or other group that may exist in the particular peptide in place of the aforementioned NH_2 group. Similarly, "COOH" refers to the free carboxy group that may be present at the carboxy terminus of a peptide, although for purposes of this invention, the "carboxy terminus" of a peptide refers to the left-most terminal amino acid residue of the peptide when represented in writing as a linear sequence of amino acid residues regardless of the reactive or other group that may exist in the particular peptide in place of the aforementioned COOH group. In keeping with standard polypeptide nomenclature, the conventional one- and three-abbreviations for the twenty amino acid residues found in proteins in nature are as follows: Y tyrosine (Y; Tyr); glycine (G; Gly); phenylalanine (F; Phe); methionine (M; Met); alanine (A; Ala); serine (S; Ser); isoleucine (I; Ile); leucine (L; Leu); threonine (T; Thr); valine (V; Val); proline (P; Pro); lysine (K; Lys); histidine (H; His); glutamine (Q; Gln); glutamic acid (E; Glu); tryptophan (W; Trp); arginine (R; Arg); aspartic acid (D; Asp); asparagine (N; Asn); and cysteine (C; Cys). Herein, all sequences of amino acid residues are represented by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus.

[095] The peptides described herein are sufficiently small to be chemically synthesized. General chemical syntheses for preparing these peptides are described hereinafter, including chemical synthesis using solid-phase strategies, partial solid-phase techniques, fragment condensation, or classical solution couplings. The peptides of the invention can also be produced by recombinant DNA techniques well known in the art. They may also be produced by the chemical or enzymatic cleavage of larger polypeptides molecules, after which the peptides of the invention are isolated and purified.

[096] In conventional solution phase peptide synthesis, the peptide chain can be prepared by a series of coupling reactions in which constituent amino acids are added to the growing peptide chain in the desired sequence. Use of various coupling reagents, e.g., dicyclohexylcarbodiimide or diisopropylcarbonyldimidazole, various active esters, e.g., esters of N-hydroxyphthalimide or N-hydroxy-succinimide, and the various cleavage reagents, to carry out reaction in solution, with subsequent isolation and purification of intermediates, is well known classical peptide methodology. See, for example, U.S. pat. no. 4,105,60. The fragment condensation method of synthesis is exemplified in U.S. pat. no. 3,972,859.

[097] Common to such chemical syntheses is the protection of the labile side chain groups of the various amino acid moieties with suitable protecting groups that prevents a chemical reaction from occurring at that site until the group is ultimately removed. Also common is the protection of an alpha amino group on an amino acid or a fragment while that entity reacts at the carboxyl group, followed by the selective removal of the alpha-amino protecting group to allow subsequent reaction to take place at that location. Accordingly, a common step in such a synthesis is the production of an intermediate compound is produced which includes each of the amino acid residues located in its desired sequence in the peptide chain with appropriate side-chain protecting groups linked to various ones of the residues having labile side chains.

[098] As far as the selection of a side chain amino protecting group is concerned, generally one is chosen which is not removed during deprotection of the alpha-amino groups during the synthesis. However, for some amino acids, e.g., His, protection is not generally necessary. In selecting a particular side chain protecting group to be

used in the synthesis of the peptides, the following general rules are followed: (a) the protecting group preferably retains its protecting properties and is not split off under coupling conditions, (b) the protecting group should be stable under the reaction conditions selected for removing the alpha-amino protecting group at each step of the synthesis, and (c) the side chain protecting group must be removable, upon the completion of the synthesis containing the desired amino acid sequence, under reaction conditions that will not undesirably alter the peptide chain.

[099] A particularly preferred synthetic technique for preparing peptides according to the invention employs the Merrifield solid-phase synthesis, although other equivalent chemical syntheses known in the art can also be used as previously mentioned. In this approach, solid-phase synthesis is commenced from the C-terminus of the peptide by coupling a protected alpha-amino acid to a suitable resin. Such a starting material can be prepared by attaching an alpha-amino-protected amino acid by an ester linkage to a chloromethylated resin (Bio Rad Laboratories (Richmond, CA)) or a hydroxymethyl resin, or by an amide bond to a benzhydrylamine (BHA) resin or para-methylbenzhydrylamine (MBHA) resin. BHA and MBHA resin supports are also commercially available, and are generally used when the desired polypeptide being synthesized has an un-substituted amide at the C-terminus. Thus, solid resin supports may be any of those known in the art.

[0100] The C-terminal amino acid, protected by Boc or Fmoc chemistry and by a side-chain protecting group, if appropriate, can be first coupled to a chloromethylated resin. Following the coupling of the BOC-protected amino acid to the resin support, the alpha-amino protecting group is removed, for example, by using trifluoroacetic acid (TFA) in methylene chloride or TFA alone. Other standard cleaving reagents, such as HCl in dioxane, and conditions for removal of specific alpha-amino protecting groups, may also be used. After removal of the alpha-amino-protecting group, the remaining alpha-amino- and side chain-protected amino acids are coupled step-wise in the desired order to obtain a peptide intermediate. As an alternative to adding each amino acid separately in the synthesis, some of them may be coupled to one another prior to addition to the solid phase reactor. Selection of an appropriate coupling reagent is within the skill of the art. The activating reagents used in the solid phase synthesis of the peptides are also well known in the peptide art, and include carbodiimides, such as N,N'-diisopropylcarbodiimide and N-ethyl-N'-(3-

dimethylaminopropyl)carbodiimide. Coupling reactions can be performed automatically, as on a Beckman 990 automatic synthesizer, programmed to generate the desired peptide.

[0101] After a peptide containing the desired amino acid sequence has been synthesized, the intermediate peptide can be removed from the resin support by treatment with a reagent, such as liquid hydrogen fluoride or TFA (if using Fmoc chemistry), which not only cleaves the peptide from the resin but also cleaves all remaining side chain protecting groups and also the alpha-amino protecting group at the N-terminus if it was not previously removed to obtain the peptide in the form of the free acid. If Met is present in the peptide, the Boc protecting group is preferably first removed using trifluoroacetic acid (TFA)/ethanedithiol prior to cleaving the peptide from the resin with HF to eliminate potential S-alkylation. When using hydrogen fluoride or TFA for cleaving, one or more scavengers such as anisole, cresol, dimethyl sulfide and methylethyl sulfide are preferably included in the reaction vessel.

[0102] If desired, cyclization of a linear peptide is preferably affected, as opposed to cyclizing the peptide while a part of the peptido-resin, to create bonds between Cys residues. To effect such a disulfide cyclizing linkage, fully protected peptide can be cleaved from a hydroxymethylated resin or a chloromethylated resin support by ammonolysis, as is well known in the art, to yield the fully protected amide intermediate, which is thereafter suitably cyclized and deprotected. Alternatively, deprotection, as well as cleavage of the peptide from the above resins or a benzhydrylamine (BHA) resin or a methylbenzhydrylamine (MBHA), can take place, followed by oxidation as described above.

[0103] The peptides of the invention may also be synthesized using an automatic synthesizer. For example, amino acids are sequentially coupled to an MBHA Rink resin (typically 100 mg of resin) beginning at the C-terminus using an Advanced Chemtech 357 Automatic Peptide Synthesizer. Couplings are carried out using 1,3-diisopropylcarbodimide in N-methylpyrrolidinone (NMP) or by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and diethylisopropylethylamine (DIEA). The FMOC protecting group is removed by treatment with a

20% solution of piperidine in dimethylformamide (DMF). Resins are subsequently washed with DMF (twice), followed by methanol and NMP.

[0104] A skilled artisan will be able to determine suitable variants of peptides of the invention using well-known techniques. For identifying suitable areas of the molecule that may be changed without destroying biological activity (*i.e.*, TLR binding, immunogenicity, etc.). For example, for similar peptides with similar activities from the same species or other species, one may compare the peptides' respective amino acid sequences to identify residues and portions of the molecules that are conserved among similar polypeptides. It will be appreciated that changes in areas of the molecule that are not conserved among similar peptides would be less likely to adversely affect biological activity and/or structure of a peptide. Similarly, the residues required for binding to TLRs may be identified (*e.g.*, by structural analyses), and may be modified to alter (positively or negatively) binding characteristics. Those skilled in the art also know that, even in relatively conserved regions, chemically similar amino acids may be substituted for the naturally occurring residues while retaining activity, albeit perhaps at reduced or enhanced levels. Therefore, even areas that may be important for biological activity can be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the peptide structure in the context of TLR binding.

[0105] Other preferred peptide variants include those containing modified amino acid side chains. Modifications may be introduced before or after peptide synthesis. Such modifications include glycosylation, or peptides having different glycosylation as compared to other peptides of the same or similar amino acid sequence. Glycosylation includes N-linked and/or O-linked glycosylation. N-linked glycosylation sites are characterized by the sequence Asn-X-Ser or Asn-X-Thr, wherein the amino acid residue designated as X may be any amino acid residue except proline. The substitution of amino acid residues to create such a site provides a potential new site for the addition of an N-linked carbohydrate chain. Alternatively, substitutions that eliminate such a sequence may result in a peptide lacking an N-linked carbohydrate chain at that site. To affect O-linked glycosylation, one would modify serine and/or threonine residues. Other modifications include those that stabilize the peptide in blood or otherwise affect the peptide's administration, distribution, metabolism, and/or excretion after administration to a patient.

[0106] Additional preferred variants include cysteine variants, wherein one or more cysteine residues are deleted or substituted with another amino acid (*e.g.*, serine) as compared to the original sequence. Cysteine variants may be useful in the context of refolding or side-chain-mediated polymerization of peptide molecules (of the same or different peptide species).

[0107] Modifications of a peptide of the invention can also be made by introducing one or more mutations in a nucleic acid molecule encoding the particular peptide such that a particular codon is changed to a codon which codes for a different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting peptide in a non-conservative manner (*i.e.*, by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (*i.e.*, by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Conservative changes generally lead to less change in the structure and function of the resulting peptide, while a non-conservative change is more likely to alter the structure, activity, or function of the resulting peptide. The preferred embodiments of the present invention should be considered to include peptides based on any one of the peptides having an amino acid sequence selected from among Seq. Id. Nos. 1-73 and which contain one conservative amino acid substitutions in the indicated sequence and/or one or additional amino acid residues at either or both of the amino- and/or carboxy-termini of the peptide. It will be understood that the invention also concerns peptides including the insertion of one or more amino acid residues between two residues listed in the peptides represented by Seq. Id. Nos. 1-73, as well as peptides wherein one or more of the amino acid residues listed in the peptides represented by Seq. Id. Nos. 1-73 has been deleted.

[0108] In other embodiments, a peptide of the invention may be fused to one or more additional peptide or polypeptide species. Such fusions typically result from the linkage of one terminus (*e.g.*, a C-terminus) of a peptide to a terminus (*e.g.*, an N-terminus) of another peptide (of the same of different peptide species) or polypeptide, directly or through a linker. In preferred embodiments, the peptides of the invention may be linked indirectly through a peptide linker that may be cleaved enzymatically

or physically (*e.g.*, by chemistry, light, *etc.*). In other preferred embodiments, a peptide (or series of peptides linked to form a polypeptide) contains a series of amino acid residues that facilitate purification. Such fusions can be made either at the amino- or carboxy terminus, directly with no linker or adapter molecule or indirectly may be through a linker or adapter molecule. A linker or adapter molecule preferably is one or more amino acid residues, typically from about 20 to about 50 amino acid residues. As described above, a linker or adapter molecule may also be designed to include a cleavage site for a protease to allow for the separation of the fused moieties. It will be appreciated that once constructed, fusion peptides can further be derivatized, as described elsewhere herein. Preferred fusion segments include, for example, metal binding domains (*e.g.*, a poly-histidine segment), immunoglobulin binding domains, sugar binding domains, or another "tag" domain that can be used to isolate the fused molecules. Such tags are typically fused to the peptide upon expression (or other synthesis), and can provide for subsequent affinity purification of the peptide of interest, for example, by column chromatography wherein the column matrix contains molecules that specifically react with the tag moieties. If desired, the tags can subsequently be removed from the peptide of interest, for example, by use of a peptidase that cleaves a sequence engineered into the linker to yield as a product a peptide of the invention.

[0109] In certain embodiments, it may be advantageous to combine one or more molecular species of the invention with one or more co-stimulatory component(s) such as cell surface proteins, cytokines, or chemokines, in one composition or as separate compositions to be delivered as part of single therapeutic regimen. The co-stimulatory component may be included in a composition of the invention in an active form, as a precursor that becomes activated following administration to a subject, or, in the case of defined polypeptide, as part of an expression vector (including in some embodiments the same expression vector that directs the expression of a peptide of the invention). Such combinations may also be delivered to a subject substantially simultaneously via the same or different routes. Alternatively, a compositions comprising a co-stimulatory component may be delivered before or after a composition comprising one or molecular species according to the invention.

[0110] Preferred co-stimulants are those that affect the innate and/or adaptive arms of the immune system, including polypeptides B7.1 and B7.2; CD5, CD9, CD2, and

CD40, and their respective ligands; polypeptides that bind members of the integrin family (*e.g.*, LFA-1 (CD11a/CD18), including members of the ICAM family (*e.g.*, ICAM-1, -2 or -3); polypeptides that bind CD2 family members; polypeptides which bind heat stable antigen (HSA or CD24); polypeptides which bind to members of the TNF receptor (TNFR) family (*e.g.*, 4-1BB (CD137), OX40 (CD134), and CD27), such as 4-1BBL (4-1BB ligand), TNFR-associated factor-1 (TRAF-1; 4-1BB ligand), TRAF-2 (4-1BB and OX40 ligand), TRAF-3 (4-1BB and OX40 ligand), OX40L (OX40 ligand), TRAF-5 (OX40 ligand), and CD70 (CD27 ligand), in addition to TNF and antibodies to TNF. Other co-stimulatory, or "adjuvant", molecules (and nucleic acid molecules encoding them) include cytokines and chemokines. Representative cytokines include, for example, interleukin-2 (IL-2), IL-4, IL-7, IL-12, IL-16, IL-18, GM-CSF, tumor necrosis factor-alpha (TNF-α), or interferons such as IFN-α, INF-γ, and consensus interferon. Other cytokines that enhance the effect of a molecule of the invention may also be used as an adjuvant. Inded, as those in the art in will appreciate, any molecule now known or later developed that potentiates enhances the activity of a molecule of the invention can be used in combination therewith. Other adjuvants include those that employ alum, calcium phosphate, Freund's adjuvant, and others known in the art.

[0111] It should be possible to prepare many, or even all, of the peptides of the invention using recombinant DNA technology. Thus, in certain embodiments, a nucleic acid molecule encoding a TLR-binding peptide is utilized. The nucleic acid molecule may comprise or consist of a nucleotide sequence encoding one or more TLR-binding peptides. The term "nucleic acid molecule" refers to a DNA or RNA molecule, as well as molecules that made from or which include known base analogs of nucleotides and nucleosides from which DNA and RNA molecules are synthesized in nature. Preferably, the nucleic acid molecules of the present invention are substantially free from other contaminating nucleic acid molecule(s) or other contaminants that might interfere with its use in peptide production or for therapeutic or diagnostic applications.

[0112] In preferred embodiments of the present invention, vectors are used to transfer a desired nucleic acid molecule encoding one or more peptides (of the same or different peptide species) of the invention to a population of recipient, or "host", cells, for example, to generate peptide species according to the invention, either for

isolation and subsequent use or directly for therapeutic purposes. A vector is any molecule used for this purpose. Typically, a "vector" is a replicon, such as plasmid, phage, or cosmid, to which another nucleic acid molecule may be attached so as to bring about the replication of the other nucleic acid molecule. In certain cases, an expression vector is utilized. An expression vector is a nucleic acid molecule that is suitable for transformation of a host cell and contains nucleic acid sequences that direct and/or control the expression of the desired nucleic acid molecule, *e.g.*, a heterologous nucleic acid encoding a TLR-binding peptide according to the invention. Expression includes, but is not limited to, processes such as transcription, translation, and splicing (if introns are present). Expression vectors typically comprise one or more flanking regions operably linked to a heterologous nucleic acid molecule encoding a one or more peptides, be they duplicative of amino acid sequences of portions of larger polypeptides found in nature, analogs or variants of such peptides, or peptides that have been synthesized or engineered de novo. Flanking sequences may be homologous (*i.e.*, from the same species and/or strain as the host cell), heterologous (*i.e.*, a region of a nucleic acid molecule that is an identifiable region within a larger nucleic acid molecule that is not found in association with the larger molecule in nature, or which itself is not found in nature), hybrid (*i.e.*, a combination of flanking sequences from more than one source), or synthetic, for example.

[0113] A flanking sequence is preferably capable of effecting the replication, transcription, and/or translation of the coding sequence carried by the desired nucleic acid molecule inserted into the vector, and is operably linked to the coding sequence. As used herein, in the context of nucleic acids, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. For instance, a promoter (*i.e.*, a regulatory region capable of being bound by an RNA polymerase and from which transcription of a downstream coding sequence is initiated) or enhancer is operably linked to a molecule carrying a coding sequence if it affects the transcription of the coding sequence. As will be appreciated, a flanking sequence need not necessarily be contiguous with the coding sequence, so long as it functions as intended. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter and the coding sequence. Similarly, an enhancer sequence may be located upstream or downstream from the coding sequence and affect transcription of the sequence.

[0114] In certain embodiments, among the flanking sequences carried by the vector is one or more transcriptional regulatory regions for driving high-level gene expression of a recombinant nucleic acid molecule encoding a peptide according to the invention in target cells. The transcriptional regulatory region may comprise, for example, a promoter, enhancer, silencer, repressor element, or combinations thereof. The transcriptional regulatory region may be either constitutive, tissue-specific, cell-type specific (*i.e.*, the region is drives higher levels of transcription in a one type of tissue or cell as compared to another), or regulatable (*i.e.*, responsive to interaction with a compound that induces or represses activity of the transcriptional regulatory region). The source of a transcriptional regulatory region may be any prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the flanking sequence functions in a cell by causing transcription of a nucleic acid within that cell. A wide variety of transcriptional regulatory regions may be utilized in practicing the present invention.

[0115] Suitable transcriptional regulatory regions include a CMV promoter (*e.g.*, the CMV-immediate early promoter), promoters from eukaryotic genes, the major early and late adenovirus gene promoters, the SV40 early promoter region, and prokaryotic promoters such the beta-lactamase promoter or phage promoters such the T7 and SP6 promoters. Other suitable promoters now known in the art or later developed may also be employed, depending upon the intended application, including those that are inducible as well as tissue- and/or cell-specific transcriptional control regions.

[0116] Expression vectors encoding a peptide according to the invention may be administered to patients by any of several available techniques. Various viral vectors that have been successfully utilized for introducing a nucleic acid to a host include retroviruses, adenoviruses, adeno-associated viruses (AAV), herpes virus, and poxvirus, among others available to those in the art. The vectors of the present invention may be constructed using standard recombinant techniques widely available to one skilled in the art. Such techniques may be found in common molecular biology references such as Molecular Cloning: A Laboratory Manual (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), Gene Expression Technology (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego,

Calif.), and PCR Protocols: A Guide to Methods and Applications (Innis, et al. 1990. Academic Press, San Diego, Calif.).

[0117] Preferred retroviral vectors include those that are lentivirus derivatives, as well as derivatives of murine or avian retroviruses. Examples of suitable retroviral vectors have been derived, for example, from Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), SIV, BIV, HIV, and Rous Sarcoma Virus (RSV). A number of retroviral vectors can incorporate multiple exogenous nucleic acid molecules. As recombinant retroviruses are preferably replication defective, they require assistance in order to produce infectious vector particles. This assistance can be provided by, for example, helper cell lines encoding retrovirus structural genes. Suitable helper cell lines include the T2, PA317, and PA12 cell lines, among others. Techniques for making infectious, replication-defective retroviral vectors are widely known in the art and can readily be adapted for use in the context of the invention.

[0118] Adenoviral vectors have proven especially useful for gene transfer into eukaryotic cells, the study eukaryotic gene expression, vaccine development, and in animal models. Routes for administrating recombinant adenoviral vectors to different tissues in vivo have included intratracheal instillation, injection into muscle, peripheral intravenous injection, and stereotactic inoculation to brain, among others. Adeno-associated virus (AAV) demonstrates high-level infectivity, broad host range, and specificity in integrating into the host cell genome. Herpes Simplex Virus type-1 (HSV-1) is yet another attractive vector system, especially for use in the nervous system because of its neurotropic properties. Poxviruses, including vaccinia, NYVAC, avipox, fowlpox, canarypox, ALVAC, and ALVAC(2), among others, may also be adapted as useful expression vectors, as is known in the art. Poxviruses shown to be useful include vaccinia, NYVAC, TROVAC, avipox, fowlpox, canarypox, ALVAC, and ALVAC(2), among others.

[0119] Non-viral expression vectors may also be used in practicing the present invention. Preferred plasmid vectors are compatible with bacterial, insect, and/or mammalian host cells. Such vectors include, for example, PCR-II, pBSII, pET15, pGEX, pEGFP-N2, pETL, pCR3, and pcDNA3.1, and many are available from commercial sources (*e.g.*, from Invitrogen, San Diego, CA; Stratagene, La Jolla, CA;

Novagen, Madison, WI; Pharmacia Biotech, Piscataway, NJ; Clontech, Palo Alto, CA). Suitable nucleic acid delivery techniques include DNA-ligand complexes, adenovirus-ligand-DNA complexes, direct injection of DNA, $CaPO_4$ precipitation, gene gun techniques, electroporation, and colloidal dispersion systems, among others. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes, with liposomes being preferred. The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used.

[0120] The TLR-binding molecules of the invention can also be used to isolate from a biological sample (*e.g.*, a cell culture, blood, tissue, *etc.*) cells that express on the outer surface of their cell membranes a TLR molecules reactive with the molecular species. If desired, these cells can be analyzed to determine the presence of a disease state in a subject, to predict prognosis, or to determine the effectiveness of a treatment regimen. Assays useful in this regard include expression profiling. The level of expression of the genes or proteins being assayed for may then be correlated with base levels to determine whether a particular disease or disorder is present within the subject (*e.g.*, a human patient), the subject's prognosis, or whether a particular treatment regimen is effective. Alternatively, cells isolated in this manner may be cultured, treated if desired, and administered to a subject (or re-administered if originally obtained from the subject).

[0121] The present invention, in another aspect, relates to compositions, including pharmaceutical compositions, comprising an effective amount of a TLR-binding molecule (*e.g.*, a TLR-binding peptide or peptidomimetic) described herein, or an expression vector encoding a TLR-binding peptide of the invention, and a physiologically acceptable carrier.

[0122] Administration of a composition of the present invention to a host may be accomplished using any of a variety of techniques known to those of skill in the art. The composition(s) may be processed in accordance with conventional methods of pharmaceutical compounding techniques to produce medicinal agents (*i.e.*, medicaments or therapeutic compositions) for administration to subjects, including

humans and other mammals, *i.e.*, "pharmaceutical" and "veterinary" administration, respectively. See, for example, the latest edition of Remington's Pharmaceutical Sciences (Mack Publishing Co., Easton, PA).

[0123] The TLR-binding molecules of the invention may be prepared as free acids or bases, which are then preferably combined with a suitable compound to yield a pharmaceutically acceptable salt. The expression "pharmaceutically acceptable salts" refers to non-toxic salts formed with nontoxic, pharmaceutically acceptable inorganic or organic acids or inorganic or organic bases. For example, the salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric, and the like, as well as salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, pamoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicyclic, sulfanilic, fumaric, methanesulfonic, and toluenesulfonic acid and the like. Salts also include those from inorganic bases, such as ammonia, hydroxyethylamine and hydrazine. Suitable organic bases include methylamine, ethylamine, propylamine, dimethylamine, diethylamine, trimethylamine, triethylamine, ethylenediamine, hydroxyethylamine, morpholine, piperazine, and guanidine.

[0124] In any event, the therapeutic compositions are preferably made in the form of a dosage unit containing a given amount of a TLR-binding molecule according to the invention and a carrier (*i.e.*, a physiologically acceptable excipient). What constitutes a therapeutically effective amount of a TLR-binding molecule for a human or other mammal (or other animal) will depend on a variety of factors, including, among others, the type of disease or disorder, the age, weight, gender, medical condition of the subject, the severity of the condition, the route of administration, and the particular compound employed. Thus, dosage regimens may vary widely, but can be determined routinely using standard methods. In any event, an "effective amount" of a TLR-binding molecule is an amount that elicits the desired immune modulation (*e.g.*, induction or enhancement of an immune response sought to be induced or enhanced or, alternatively, the reduction or prevention of an immune response sought to be reduced or prevented). The quantity of a TLR-binding molecule required to achieve the desired effect will depend on numerous considerations, including the particular TLR-binding molecule, the disease or disorder to be treated, the capacity of the subject's immune system to respond to the TLR-binding molecule, route of

administration, and degree of immune modulation desired. Precise amounts of the TLR-binding molecule required to achieve the desired effect will depend on the judgment of the practitioner and are peculiar to each individual subject. However, suitable dosages may range from about several nanograms (ng) to about several milligrams (mg) of active ingredient per kilogram body weight per day. Suitable regimens for initial administration and one or more booster administrations are also variable.

[0125] The preparation of therapeutic compositions is well understood in the art. Typically, such compositions are prepared as injectable, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients that are physiologically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water for injection, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, anti-pyretics, stabilizing agents, thickening agents, suspending agents, anesthetics, preservatives, antioxidants, bacteriostatic agents, analgesics, pH buffering agents, *etc.* that enhance the effectiveness of the active ingredient. Such components can provide additional therapeutic benefit, or act towards preventing any potential side effects that may be posed as a result of administration of the pharmaceutical composition.

[0126] The compositions of the invention may be administered orally, parentally, by inhalation spray, rectally, intranodally, or topically in dosage unit formulations containing conventional carriers, adjuvants, and vehicles. In the context of therapeutic compositions intended for human administration, pharmaceutically acceptable carriers are used. The terms "pharmaceutically acceptable carrier" and "physiologically acceptable carrier" refer to molecular entities and compositions that are physiologically tolerable and do not typically produce an unintended allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a subject and are thus suitable for accomplishing or enhancing the delivery of a TLR-binding molecule as a medicament.

[0127] For oral administration, the composition may be of any suitable form, including, for example, a capsule, tablet, suspension, or liquid, among others. Liquids may be administered by injection as a composition with suitable carriers including saline, dextrose, or water. The term "parenteral" includes infusion (including continuous or intermittent infusion) and injection via a subcutaneous, intravenous, intramuscular, intrasternal, or intraperitoneal route. Suppositories for rectal administration can be prepared by mixing the active ingredient(s) with a suitable non-irritating excipient such as cocoa butter and/or polyethylene glycols that are solid at ordinary temperatures but liquid at physiological temperatures.

[0128] The compositions may also be prepared in a solid form (including granules, powders or suppositories). The compositions may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers *etc.* Solid dosage forms for oral administration may include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound may be admixed with at least one inert excipient such as sucrose, lactose, or starch. Such dosage forms may also comprise additional substances other than inert diluents, *e.g.*, lubricating agents such as magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings. Liquid dosage forms for oral administration may include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions may also comprise adjuvants, such as wetting sweetening, flavoring, and perfuming agents.

[0129] Injectable preparations, such as sterile injectable aqueous or oleaginous suspensions, may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Suitable vehicles and solvents that may be employed are water for injection, Ringer's solution, and isotonic sodium chloride solution, among others. In addition, sterile, fixed oils can be employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed,

including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

[0130] For topical administration, a suitable topical dose of a composition may be administered one to four, and preferably two or three, times daily. The dose may also be administered with intervening days during which no dose is applied. Suitable compositions for topical delivery often comprise from 0.001% to 10% w/w of active ingredient, for example, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation. Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes), and drops suitable for administration to the eye, ear, or nose.

[0131] Exemplary methods for administering the compositions of the invention (*e.g.*, so as to achieve sterile or aseptic conditions) will be apparent to the skilled artisan. Certain methods suitable for such purposes are set forth in Goodman and Gilman's The Pharmacological Basis of Therapeutics, 7th Ed. (1985). The administration to the patient can be intermittent; or at a gradual, continuous, constant, or controlled rate.

[0132] The invention also contemplates kits comprising a composition of the invention stored in suitable container (*e.g.*, a glass vial, ampoule, or disposable injection device) packaged in a box in conjunction with a package insert describing, among other things, the disease or order for which the composition is used as a treatment and how to administer the composition. Containers may be designed for a single use (*e.g.*, as a unit dose in a sealed vial to which water for injection may be added by a needle through, for example, a sealed rubber stopper), or for multiple uses. As will be appreciated, when a composition is intended for injection, the composition may be stored in dry form and is reconstituted using a suitable diluent just prior to administration. In such instances, a kit will also preferably include a container containing a suitable carrier, diluent, or excipient for reconstitution of the dry composition. Additionally, the kit can include instructions for mixing or combining ingredients and/or administration and such other information as may be desired or required by law.

EXAMPLES

[0133] The following Examples are provided to illustrate certain aspects of the present invention and to aid those of skill in the art in practicing the invention. These Examples are in no way to be considered to limit the scope of the invention in any manner.

EXAMPLE I: Selection of Peptide: eHSP60p1

[0134] A Data Summary Table was produced for eHSP60p1. The primary amino acid sequence of *E. coli* HSP60 was analyzed using the combination of four different algorithms described above. The result of this analysis was displayed in a Data Summary Table I that shows region 426-444 of the primary amino acid structure of *E. coli* HSP60. Of the entire sequence analysis, regions were selected that had a "Helix-Turn-Helix" predicted structure. In italics is highlighted the β-turn region. In plain print the flanking regions having a predicted α-helix structure.

Data Summary Table I

Pos.	AA	AI	KD hyd	SurfP	CFhlx	CFsht	CFturn	CFval	CFtype
426	L	-0.315	-0.429	0.434	1.181	0.897	0.386	0.65	H
427	A	-0.233	0.471	0.535	1.134	0.910	0.291	0.65	H
428	D	-0.249	0.414	0.490	1.097	0.906	0.423	0.65	H
429	L	-0.241	0.357	0.463	1.103	0.910	0.455	0.65	H T
430	R	-0.117	1.400	0.536	1.046	0.873	0.283	0.65	H T
431	*G*	*0.190*	*2.157*	*0.614*	*1.067*	*0.851*	*0.455*	*0.65*	*H T*
432	*Q*	*0.208*	*2.157*	*0.614*	*1.067*	*0.851*	*0.315*	*0.65*	*T*
433	*N*	*0.507*	*3.200*	*0.695*	*1.063*	*0.820*	*0.498*	*0.65*	*T*
434	*E*	*0.481*	*3.057*	*0.666*	*1.054*	*0.787*	*0.294*	*0.65*	*T*
435	*D*	*0.221*	*2.400*	*0.668*	*1.104*	*0.869*	*0.489*	*0.65*	*T*
436	*Q*	*0.198*	*1.957*	*0.580*	*1.003*	*0.886*	*0.335*	*0.90*	*T*
437	*N*	*0.072*	*0.814*	*0.500*	*1.013*	*0.984*	*0.455*	*0.90*	*T*
438	*V*	*0.067*	*0.871*	*0.528*	*0.983*	*0.987*	*0.255*	*0.90*	*T*
439	G	-0.293	-0.229	0.450	0.964	1.097	0.261	0.65	H
440	I	-0.378	-0.986	0.371	0.967	1.111	0.242	0.65	H
441	K	-0.553	-2.029	0.298	1.024	1.149	0.208	0.25	H
442	V	-0.502	-0.786	0.405	1.031	1.077	0.259	0.00	H
443	A	-0.531	-1.100	0.415	1.136	1.074	0.242	0.00	H
444	L	-0.464	-0.729	0.424	1.207	1.006	0.257	0.25	H

[0135] Next, a Data Plot was generated wherein the same region of the *E. coli* HSP60 protein sequence originated by the same computer analysis was performed using a combination of the four different algorithms described above (See Figs. 1-4). This analysis confirmed a "core sequence" including the amino acids 429-438 that

was predicted to be surface exposed by the four different algorithms and matched with the "Helix-Turn-Helix" predicted structure by the Chou-Fasman analysis.

[0136] The combination of Summary Table and Data Plot provided for prediction and identification of the regions of the protein that have the highest surface exposure prediction. For the selection of the peptides predicted to play a role in interacting with TLR on monocytes and monocytes, regions of the proteins were selected that had high scores for all the three selected algorithms, *i.e.* -Antigenic Index; -Kite-Doolittle Hydrophilicity; -Surface Probability for the "core region". Flanking regions had less surface exposure prediction score values and that satisfied the rule of "Helix-Turn-Helix" structure.

[0137] Additionally, the selection found by the above analysis was "refined" by re-analyzing the selected region, *i.e.* peptide, alone using the same algorithms and comparing these predictions with the same region within the original protein. If changes occurred in the score values, changes of the amino acids of the flanking ends were made to make sure that the two predictions were as close as possible and maintained or acquired the "Helix-Turn-Helix" structure. This refinement produced the affect on Antigenic Index, hydrophilicity, Surface Probability, and Chou-Fasman as shown in Figures 5-8 wherein region 426-444 of the primary amino acid structue of *E. coli* HSP60 is reanalyzed using the combination of four different algorithms described above.

[0138] As an example of such refinement, the invention peptides include derivative peptides of wild-type peptides such as P2 and P6 disclosed in Table I. These single amino acid changes were brought about because in the computer analysis these substitutions generated a "predicted-structure" more likely to be surface-exposed.

[0139] Further refinement of the peptide selection was carried out (based on computer models if available). For example, a three dimensional computer model of *E. coli* HSP60 protein was used to examine the position of the P1 peptide. Selected regions were originated using as source the structural coordinates of the crystallized *E. coli* protein Groel (otherwise called HSP60) and visualized using Cn3D4. This software is a visualization tool for biomolecular structures, sequences, and sequence alignments and is publicly available at website address: www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml. In yellow is highlighted the

region 426-444 of the *E. coli* HSP60 protein. It can be noted that the region 426-444, as predicted, is surface exposed (See Figure 9).

EXAMPLE 2: Effect of HSP-derived peptides on immune modulation

[0140] HSP-derived peptides that bind TLR receptors can affect expression of TLR2, TLR4 receptor and secretion of TNF-alpha and IL-6 proinflammatory cytokines, on monocyte-derived dendritic cells. Here, human heat shock protein 60 (hHSP60) is shown to elicit a potent pro-inflammatory response in cells of the innate immune system and therefore has been proposed as a danger signal of stressed or damaged cells. Previous work showed that mouse or human macrophages, as well as endothelial or smooth muscle cells, were found to elicit a pro-inflammatory response when incubated with recombinant hHSP60. The response included the up-regulation of adhesion molecule expression and the release of inflammatory mediators such as IL-6 and TNF-alpha. Since autologous hsp60 may be aberrantly expressed on the cell surface in response to stress and will be set free from the cell interior during necrosis, these findings point to a role of hHSP60 in initiating or sustaining Th1- dependent tissue inflammation. Interestingly, microbial HSP60/65 also induces a pro-inflammatory response in innate immune cells suggesting that damaged autologous cells and microbial pathogens may alert innate immunity via the same recognition system. TLR4 receptor has been identified to mediate HSP60 signaling while TLR2 appears to be important in LPS binding and signaling. It was decided to investigate if peptides derived from phylogenetically separate hsp60 species would interact with innate immune cells via different recognition pathways as TLr2 and TLr4. As shown in Figure **10A-D**, monocyte-derived dendritic cells respond to the different peptides with a kinetics that revealed peak levels of expression at 6 hours after stimulation. P1, P3, and P4 were able to induce, at various levels, expression of TLR2 and TLR4 receptor. Also TNF-alpha and IL-6 cytokine secretion was measured with P1, P3 and P4. These data indicate correlation between receptor expression and pro-inflammatory cytokines secretion. TNF-alpha and IL-6 production was induced with P2 at 6 hrs (Fig. **10C-D**) but very low level of Tlr2 and Tlr4 receptor expression was detected (Fig. **10A-B**). This result may be due to a different activation pathway by the P2 peptide compare to the previous peptides. Interestingly, P1 was affecting

TLR2 and TLR4 expression at 48 hrs and not at 24 hrs (Fig. **10A-B**), while P4 was giving TLR4 expression up to 24 hrs after stimulation (Fig. **10B**) but no cytokine secretion was measurable. As a positive control, HSP60 was used for all parameters analyzed (Figs. **10A-D**) while LPS was used for TNF-alpha and IL-6 induction.

Materials and Methods

[0141] Monocytes and DC preparation

[0142] PBMC were derived by Ficoll-Hypaque density gradient centrifugation from buffy coats of healthy blood donors obtained from the San Diego Blood Bank. CD14-positive monocytes were enriched to 93% purity by Percoll density gradient centrifugation. DC were generated by previously isolated monocytes in IMDM medium supplemented with 1% human AB serum, GM-CSF and IL-4 for 6 days, replacing 50% of the culture medium every other day.

[0143] Induction of TLR2, TLR4 and cytokine release from monocyte-derived dendritic cells

[100] DC (1x106 cells/well) were stimulated with the different peptides and as a control HSP and LPS were used. Cells were harvested respectively at 6, 24, 48, and 72 hours.

Flow cytometry assay.

[101] Specific cell surface staining was performed using FITC, PE and Cy-conjugated antibody (Ab) in saturating amounts. Specific isotype controls were used for each sample. Anti-TLR-2, anti-TLR-4, anti-CD86, anti-CD83, anti-HLA-DR, anti-TNF-α and anti-IL-6 Abs were used. Results were expressed as the percent of positive cells for a given marker. The relative percent of fluorescence intensities was calculated as the value of the percent of fluorescence intensities of the respective marker Ab minus the specific isotype control and the values obtained with non-stimulated cells.

EXAMPLE 3: Effect of HSP-derived peptides on secretion of TNF-alpha from a mouse macrophage cell line, RAW264.7

[102] As discussed above, it was desired to investigate if peptides derived from phylogenetically separate hsp60/70 species would interact and elicit an inflammatory response in a mouse monocyte/macrophage cell line. As shown in Figure **11**, RAW264.7 cells respond to the different peptides with a kinetics that revealed peak levels of expression at 72 hours after stimulation. P10 (hsp70 human derived peptide) and P3, P4 (hsp60 human derived peptide) are inducing a very strong TNF-α secretion. Also, P1 and P2 (HSP60 *E. coli*-derived peptide) and P5, P6 (hsp60 Mycobacteria-derived peptide) are inducing a very comparable TNF-α secretion. Thus, phylogenetically separate hsp60 /70 can induce an inflammatory response, as indicated by the medically relevant mouse cell line model.

EXAMPLE 4: Diagnostic Application

[103] In this example, the peptides of the invention are used for diagnosis of patients with Autoimmune Diseases. Specifically, a panel of the selected/identified HSP-derived peptides are used to screen patient PBMC-derived Monocytes, Macrophages and Monocyte derived-dendritic cells as well as other immune cells expressing TLR. Such screening can be used to identify specific TLRs that can be then "targets" for anti-inflammatory therapy. Such TLRs can be disease specific. This then provides for autoimmune specific/tailored immune intervention based on the inhibition of innate immune mediated proinflammatory pathway activation.

[104] Natural as well as synthetic antigen-specific therapeutics derived from TLR binder peptides can be then used to interfere/inhibit the binding of HSP and their derivatives to TLRs and therefore inhibiting or interfering with the resulting triggered inflammatory response in a disease-specific fashion.

EXAMPLE 5: Therapeutic Application:

[105] Therapeutic applications comprise three general medical areas including cancers, infectious diseases and immunodeficiencies and in each of these areas, the HSP-derived peptide sequences of the invention may be identified for interacting with specific TLRs allowing additionally for the capability of designing peptides or peptidomimetics which bind directly to TLR and induce a pro-inflammatory

response. Clinical applications range from Cancer (systemic or local) to infectious diseases (including granuloma) and immunodeficiencies. In another aspect, clinical applications include use of the peptides as adjuvants in vaccine formulations against a whole range of infectious disease and cancer. In yet another aspect, the peptides of the invention can be applied to autoimmune diseases, for example, the HSP-derived peptide sequences can be identified and designed or peptidomimetics created for interacting with or binding directly to specific TLR to avoid inducing proinflammatory responses. In this instance, the invention peptides are contemplated to act in the form of, for example, molecular competitor/inhibitor of natural HSP, or triggers of DC-2-type responses (tolerogenic) by acting on and triggering different activation pathways. Further, in this example, identified HSP-derived peptide sequences interacting with specific TLR can be used for the development/identification of natural as well as synthetic antigen-specific therapeutics able to interfere/inhibit the binding of HSP and their derivatives to TLRs and therefore inhibiting or interfering with the resulting triggered inflammatory response.

[106] Inhibition or interference with the inflammatory response include the inhibition of all or part of the proinflammatory mediators such as TNF-a or IL-6 and of the Th1-promoting cytokines IL-12 and IL-15 and possibly other cytokines and mediators secreted as a results of interaction between both bacterial and human HSPs and TLR expressed on Monocytes, Macrophages and Monocyte-derived Dendritic cells as well as other immune cells expressing TLR.

[107] Natural as well as synthetic antigen-specific therapeutics derived from the HSP-derived peptides able to interfere/inhibit the binding of HSP and their derivatives to TLRs can be preferably administered. Further, peptidomimetics synthetic molecules able to block specific TLR involved in the induction of proinflammatory cytokine secretion can be designed. Further still, immunization with TLR identified in the screening assay can be accomplished to bring about lowering of inflammatory responses. Further still, associated with such therapeutic application is the production of a medicament for use in the treatment of cancers, infectious diseases and immunodeficiencies as earlier described. It is contemplated that such medicament can include components suitable for any of an injectable fluid, a topical absorbable fluid or cream, an ingestible compound or fluid, or a

suppository. It is also contemplated that such a medicament might include, as needed, pharmaceutically acceptable carriers and excipients.

* * *

[108] All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the spirit and scope of the invention as defined by the appended claims.

[109] All patents, patent applications, and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents, patent applications, and publications, including those to which priority or another benefit is claimed, are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

[110] The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.